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(54) Title: ENZYMATIC PRODUCTION OF D-AMINO ACIDS

(57) Abstract

A method for synthesizing D-alpha-amino acids by contacting under reaction conditions an alpha-keto acid corresponding to the D-alpha-amino acid, and a source of an amino group comprising an L-alpha amino acid or a DL-alpha-amino acid mixture, i.e., a racemic mixture of an amino acid, in the presence as catalyst of D-amino acid transaminase is disclosed and claimed. The method can be under batch or continuous conditions, including means for driving equilibria towards production of the D-alpha-amino acid, such as causing a reduction in the concentration of alpha-keto acid corresponding to the source of amino group by adding to the reaction medium a consumer thereof, e.g., NADH and lactate dehydrogenase, or by a physical separation.

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ENAYMATIC PRODUCTION OF D-AMINO ACIDS

BACKGROUND OF THE INVENTION

This invention relates to an enzymatic method for the synthesis of D-amino acids utilizing an L-amino acid or a racemic mixture of amino acids together with the alpha-keto acid corresponding to the D-amino acid to be produced as source materials. For example it relates to the enzymatic production of D-leucine from a mixture of DL-alanine and alpha-ketoisocaproate.

As is well known in the art, in alpha amino acids, the alpha carbon atom is a chiral center with the result that such acids, be they natural occurring or otherwise exist is D- and L- forms. Most, but not all natural amino acids, whether the 20 common amino acids or the more than 100 amino acids which are not too common exist in the L-form in their natural state. A few, for example some amino acids in the walls of certain bacteria exist in D-forms. The chemical synthesis of amino acids always results in a mixture of the DL-forms referred to as a racemic mixture.

For a discussion of amino acids attention is directed to A.L. Lehninger, Biochemistry, (Second Edition) including Chapters 4 and 21, Worth Publishers, Inc. 1978, L.F. Fieser et al, Advanced Organic Chemistry , including Chapter 31, Reinhold Publishing Corp., 1961, L. Stryer, Biochemistry, Second Edition, including Chapters 18 and 21, W.H. Freeman and Co. 1981, each of which being hereby incorporated herein by reference.

In one form of metabolism of amino acids, a transamination takes place during which one amino acid is converted to another by transamination. The reaction is well known and takes place in

the presence of a transaminase enzyme, pyridoxal or pyridoxamine phosphate and an alpha-keto acid corresponding to the alpha amino acid to be produced. A number of amino acid transaminases are known which are capable of utilizing D-amino acids or L-amino acids as substrates for the production of other D-amino acids or L-amino amino acids.

For instance, in bacteria, an enzyme called D-amino acid transaminase catalyzes the synthesis of D-amino acids from other D-amino acids. This encyme is responsible for catalyzing the synthesis of D-amino acids for the peptidoglycan moiety of the bacterial cell wall. D-amino acid transaminase can be obtained by known procedures (See Jones, W., et al (1985) Methods Enzymol. 113: 108-113, hereby incorporated herein by reference).

A thermostable D-amino acid transaminase has been isolated from Bacillus sphaericus. The gene for its activity was isolated, sequenced and cloned into the plasmid P ICT113 which was used to transform Escherichia coli to express the enzyme. The procedures for obtaining D-amino transaminase are well known and are described in detail in the articles cited herein, especially in the Examples section of this disclosure.

In the usual mechanism of action with L-amino acids and transaminases (E), an amino acid substrate donates its amino group to the pyridoxal 5′ phosphate (PLP) form of the enzyme (L-E-PLP) to generate the corresponding keto acid and the pyridoxamine 5′ phosphate form of the enzyme (L-E-PMP) (Note that the designation "L" is to show that the enzyme is specific to L-amino acids and is not meant to designate the stereoisomer of the enzyme). This is referred to as the first half-reaction. The second half-reaction involves a keto acid substrate accepting the amino group from L-E-PMP to produce the L-amino acid

corresponding to the keto acid and to regenerate L-E-PLP. For D-amino acid transaminase ("D-E"; noting again that the designation "D" is not meant to designate the stereoisomer of the enzyme), the analogous reaction pathway would be:

1) D-amino acid$_1$ + D-E-PLP $\rightleftharpoons$ alpha-keto acid$_1$ + D-E-PMP
2) Alpha-keto acid$_2$ + D-E-PMP $\rightleftharpoons$ D-amino acid$_2$ + D-E-PLP

It has now unexpectedly been discovered that D-amino acid transaminase can utilize both D-and L-amino acids as substrates, sources of amino groups.

D-amino acids have a variety of uses. For instance, D-amino acids may be used to synthesize polypeptide enzymes, antibiotics, and other physiologically active substances with a view to producing products which are less susceptible to enzymatic degradation, e.g., by capping the ends of such substances with D-amino acids so that degrading enzymes specific to L-enantiomers do not "recognize" (and consequently degrade) substances so capped with D-amino acids.

A number of useful therapeutic agents are low molecular weight peptides containing one or more D-amino acid residues. D-amino acids are useful for the synthesis of such products. These include antibiotics such as tyrocidin A which contains D-phenylalanine; actinomycin D which contains D-valine; penicillin N which contains D-alpha amino valerate; and, cephalosporin C which contains D-alpha amino valerate. The immunosuppresant cyclosporin A contains D-alanine.

Most D-amino acids are commercially available, as are the corresponding alpha-keto acids.

Techniques for producing peptides containing D-amino acids are well known, see, for example the discussions of the techniques such as classical Merrifield solid phase techniques, in, J. Am. Chem. Soc. 85: 2149 (1963), J. Org. Chem. 43: 2845 (1978), J. Am. Chem. Soc. 102: 6117 (1980), J. Am. Cem. Soc. 105: 6442 (1983), and J. Am. Chem. Soc. 108: 5242 (1986) each of which being hereby incorporated herein by reference.

SUMMARY OF THE INVENTION

It has now been surprisingly discovered that D-amino acid transaminase, especially in its active or pyridoxal 5' phosphate form, catalyzes the synthesis of D-amino acids by the transfer of amino groups from L- or DL-amino acids to alpha-keto acids corresponding to the D-amino acids being synthesized. Further, it has now been surprisingly discovered that D-amino acids can be synthesized by converting corresponding alpha-keto acids by the transfer of amino groups from L- or DL-amino acids in the presence as catalyst of D-amino acid transaminase, especially in its active or pyridoxal 5' phosphate form, under continuous or batch conditions such that in the first half-reaction the equilibrium is driven towards the production of the pyridoxamine 5' phosphate form of D-amino acid transaminase, preferably through a reduction in concentration in the reaction medium of alpha-keto acids formed during the reaction which corresponds to the L- or DL-amino acids, thereby causing in the second half-reaction the equilibrium to be driven towards the production of the desired D-amino acids, and providing high yields of the desired D-amino acids.

Thus, the present invention provides a method for synthesizing a D-alpha-amino acid which comprises contacting under selected reaction conditions an alpha-keto acid corresponding to the D-alpha-amino acid to be produced and a source of an amino group comprising an L-alpha-amino acid or a DL-alpha-amino acid mixture, in the presence as catalyst of D-amino acid transaminase in its active or pyridoxal 5′ phosphate form.

The present invention also provides a method for synthesizing a D-alpha-amino acid under either continuous or batch conditions which comprises contacting under reaction conditions an alpha-keto acid corresponding to the D-alpha-amino acid, and a source of an amino group comprising an L-alpha amino acid or a DL-alpha-amino acid mixture, in the presence as catalyst of D-amino acid transaminase in its active or pyridoxal 5′ phosphate form; said continuous or batch conditions including means for driving the equilibria towards the production of the D-alpha-amino acid; and, said means for driving the equilibria preferably comprising a means for reducing the concentration in the reaction medium of the alpha-keto acid formed during the reaction which corresponds to the L-alpha- or DL-alpha-amino acid.

DESCRIPTION OF DRAWINGS

Fig. 1 shows the effects of D-alanine and L-alanine on the spectral properties of D-amino acid transaminase.

Fig. 2 shows a coupled enzyme assay to detect formation of pyruvate from L-alanine.

Fig. 3 shows a coupled enzyme assay to detect formation of D-alanine from L-alanine.

Fig. 4 shows a determination of D-alanine and D-glutamate from L-alanine and alpha-ketoglutarate.

DETAILED DESCRIPTION

The present invention provides a method for synthesizing a D-alpha-amino acid which comprises contacting under selected reaction conditions an alpha-keto acid corresponding to the D-alpha-amino acid to be produced and a source of an amino group comprising an L-alpha-amino acid or a DL-alpha-amino acid mixture, in the presence as catalyst of D-amino acid transaminase in its active or pyridoxal 5′ phosphate form.

The present invention also provides a method for synthesizing a D-alpha-amino acid under either continuous or batch conditions which comprises contacting under reaction conditions an alpha-keto acid corresponding to the D-alpha-amino acid to be produced, and a source of an amino group comprising an L-alpha-amino acid or a DL-alpha-amino acid mixture, in the presence as catalyst of D-amino acid transaminase in its active or pyridoxal 5′ phosphate form; said continuous or batch conditions including means for driving the equilibria towards the production of the D-alpha-amino acid; and said means for driving the equilibria preferably comprising a means for reducing the concentration in the reaction medium of the alpha-keto acid formed during the first half reaction which corresponds to the L-alpha- or DL-alpha amino acid.

The present invention thus provides a method for the synthesis of a D-amino acid from a L-amino acid or racemic mixtures of an alpha-amino acid employing D-amino acid transaminase which produces high yields and ensures the fidelity of the stereospecificity of the reaction pathway.

Any D-alpha-amino acid for which there is a corresponding alpha keto acid can be synthesized by the method of the present invention. Likewise, any L-alpha- or DL-alpha-amino acid having an amino group which can be transferred can be used as the source of the amino group in the method of the present invention. A way to detect whether a particular L-alpha-amino acid or racemic mixture thereof has an amino group which can be transferred, is to observe the spectral shift of D-amino acid transaminase in the presence of the L-alpha amino acid. The absorption spectrum of D-amino acid transaminase displays two bands: one at 420nm corresponding to the pyridoxal 5′ phosphate form; and another at 338nm corresponding to the pyridoxamine 5′ phosphate (PMP) form. Addition to D-amino acid transaminase of a L-alpha-amino acid having an amino group which can be transferred results in an observable shift in the absorption spectrum from 420nm with a corresponding increase in the 338nm band, indicating that the concentration of D-amino acid transaminase in the pyridoxal 5′ phosphate form has decreased while the concentration of the enzyme in the pyridoxamine 5′ phosphate form has increased, i.e., that the D-amino acid transaminase has accepted the amino group from the L-alpha amino acid. Presently, L- or DL-alanine are preferred sources of the amino group because of easy availability and because L-alanine produces a significant shift in the absorption spectrum of D-amino acid transaminase.

Furthermore, any alpha-keto acid can be converted into a D-alpha-amino acid by the method of the present invention. Examples of alpha-keto acids which can be converted into D-alpha-amino acids by the method of the present invention include pyruvate, alpha-ketoglutarate, and alpha-ketobutyrate.

In one embodiment of the present invention under continuous conditions the active or pyridoxal 5' phosphate form of D-amino acid transaminase is absorbed on or linked to a resin column. Since D-amino acid transaminase has both amino and carboxyl groups which can be used to absorb or link D-amino acid transaminase to the resin, any resin capable of forming a peptide bond with D-amino acid transaminase or capable of bonding with D-amino acid transaminase via its amino or carboxyl groups can be employed in the method of the present invention. Examples of suitable resins include any of the standard amine resins, Affi-Gel 10, Affi-Gel 15 and Affi-Gel 10/15 which are available from Bio-Rad, Chemical Division, Richmond, California, and which are suited for coupling free amino group ligands, as well as Affi-Gel 102, Aminoethyl Bio-Gel P-2, and Amino ethyl Bio-Gel P-100, which are also available from Bio-Rad and are suited for coupling COOH ligands.

After the active or pyridoxal 5' phosphate form of D-amino acid transaminase has been absorbed on or linked to a suitable resin column, the source of an amino group comprising an L-alpha or DL-alpha amino acid, e.g., D-alanine or DL-alanine, is passed over the column, resulting in the alpha-keto acid corresponding to the L-alpha or DL-alpha amino acid, e.g., pyruvate, flowing off the column, and D-amino acid transaminase on the column being converted to the pyridoxamine 5' phosphate form.

Thereafter the alpha-keto acid corresponding to the desired D-alpha amino acid is passed over the column resulting in the desired D-alpha amino acid flowing off the column until D-amino acid transaminase on the column substantially reverts back to the pyridoxal 5′ phosphate form. A ninhydrin test will provide an indication that amino acid is flowing off the column. When D-amino acid transaminase has substantially reverted back to the pyridoxal 5′ phosphate form, the alpha-keto acid corresponding to the desired D-alpha amino acid will flow off the column without substantial conversion to the desired D-alpha amino acid, whereupon additional source of amino group is then again passed over the column to regenerate the pyridoxamine 5′ phosphate form of D-amino acid transaminase on the column and continue the conversion of the alpha-keto acid corresponding to the desired D-alpha amino acid.

This method is considered continuous because the pyridoxamine 5′ phosphate form of D-amino acid transaminase can be continually regenerated on the column and the conversion to the desired D-alpha amino acid run continuously. Further, this continuous method of the present invention can easily be automated employing instruments known in the art.

In another embodiment of the present invention, after the active or pyridoxal 5′ phosphate form of D-amino acid transaminase has been absorbed on or linked to a suitable resin column and the source of an amino group has been passed over or exposed to the column, thereby causing D-amino acid transaminase on the column to be converted to the pyridoxamine 5′ phosphate form, the column can then be soaked in the alpha-keto acid corresponding to the desired D-amino acid to be produced. In this embodiment the desired D-amino acid is produced batchwise;

D-amino acid transaminase on the column is converted to the pyridoxal 5' phosphate form as the desired D-amino acid is produced; and, the column can then be reused.

In yet another embodiment of the present invention the active or pyridoxal 5' phosphate form of D-amino acid transaminase is admixed with the source of an amino group comprising an L-alpha or DL-alpha amino acid, and the alpha-keto acid corresponding to the desired D-amino acid to be produced, and optionally a consumer of the alpha-keto acid corresponding to the L-alpha or DL-alpha amino acid which is produced during the reaction; the consumer effectively removes the alpha-keto acid corresponding to the L-alpha or DL-alpha amino acid from the reaction medium, thereby shifting the equilibria towards the production of the desired D-amino acid. A presently preferred consumer is nicotinamide adenine dinucleotide, reduced form (NADH) and lactate dehydrogenase (LDH). For instance, NADH and LDH reduce pyruvate to lactate.

The present invention can also be performed at a pH such that the desired D-amino acid is produced as an acid-salt which upon appropriate acidification yields the acid form of the desired D-amino acid.

The following examples are given by way of illustration only and are not to be considered as limitations of the present invention, many apparent variations of which are possible without departing from the spirit and scope thereof.

EXAMPLES

MATERIALS AND METHODS

Enzyme Purification and Chemicals - D-Amino acid transaminase from a thermophilic species of Bacillus was purified and characterized and the isolated enzyme was demonstrated to be homogeneous as described in: Merola, M., et al (1989) Biochemistry, 28:505-509; Martinez del Pozo, A., et al (1989) Biochemistry, 28:510-516; Tanizawa, K., et al (1989) J. Biol. Chem., 264:2445-2449; Tanizawa, K., et al (1989) J. Biol. Chem. 264:2450-2454; Stoddard, B., et al (1987) J. Mol. Biol. 196:441-442, each of which is hereby incorporated herein by reference. Amino acids, enzymes, and coenzymes used for the assays were purchased from Sigma or Ajinimoto.

D-Amino Acid Transaminase Activity Assay - The lactate dehydrogenase-coupled assay was used to monitor the production of pyruvate (See Jones, W. et al (1985) Methods Enzymol. 113: 108-113, hereby incorporated herein by reference) either from D-alanine and alpha-ketoglutarate, or from L-alanine and alpha-ketoglutarate. One unit of enzyme activity is defined as the amount of protein that produces 1 μmol of alpha-keto acid per minute at 37°C. Although the enzyme is a dimer the specific activity is given as units of activity per μmol of monomeric subunit. The specific activity in the absence of free PLP in the buffer is 1.22×10^4 units/μmol.

Determination of Amino Acid Configuration - Two procedures were used - one employed a coupled enzyme assay and the other used a chemical derivatization method. For the former procedure, D-amino acid oxidase and lactate dehydrogenase were employed to measure production of pyruvate from L- or D-alanine. The

reaction mixture (1.5 or 3.0 ml) contained 0.3 M potassium phosphate, pH 8.5, 0.3 mM NADH, 3 μg/ml of lactate dehydrogenase, 1 mM flavin adenine dinucleotide, 60 μg/ml of catalase, and 250 μg/ml of D-amino acid oxidase. This protocol was employed to check any possible contamination of the L-alanine preparations with D-alanine. L-alanine from Ajinomoto was used since it was shown by this assay that the amount of the D-enantiomer present, if any, was less than 0.002%. When this assay was used to measure the formation of D-alanine from L-alanine catalyzed by D-amino acid transaminase, these conditions were changed slightly, as described below with reference to Figures. The major modification was that 12.5 μg/ml of D-amino acid oxidase was used.

The second method to establish amino acid configuration employed 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) (Pierce) ("Marfey's reagent") (See Marfey, P. (1984) Carlsberg Res. Commun. 49:561-569; Kochbar, S. et al (1988) Eur. J. Biochem. 175:433-438, each of which is hereby incorporated herein by reference). The reaction mixture (0.5 ml) contained 0.3 M potassium phosphate, pH 8.5, 20 mM L-alanine, and 0.2 mM or 20.0 mM of keto acid and 10.8 uM enzyme (as monomer). After 1 hr at 37°C, the tubes were immersed in ice and then deproteinized with Centricon 10 devices, which were centrifuged at 5,000 g for 20 min. The relative content of the diastereoisomers of the amino acids was calculated after derivatization with Marfey's reagent and separation by high performance liquid chromatography (HPLC) on a Beckman-Altex system. The derivatized amino acids (about 40-170 nmol) were injected onto a YMC reverse phase column (C_{18}, 5 μm, 0.6 x 150 cm), equilibrated with buffer A (0.1% v/v trifluoroacetic acid in water). The column was eluted with a linear gradient of 0-12% and then 12-18% of buffer B (50% v/v n-propanol in 0.1% v/v trifluoroacetic acid) for 10 and 100 min,

respectively. The eluant was monitored at 340 nm and the relative amounts of each isomer were calculated from their peak areas and the extinction coefficient of each of the separate FDAA-amino acid derivatives prepared separately. To investigate the possible production of L-glutamate from L-alanine, it was necessary to use a longer column (Altex Ultrasphere ODS column C_{18}, 5 µM, 0.5 x 25 cm) and a different buffer B, which contained 40% rather than 50% n-propanol. The gradient and elution conditions used were the same as described above.

Other Analytical Determinations - The absorption spectra were determined on an Aminco DW-2 spectrophotometer. The protein concentration of the transaminase solutions was determined by performing amino acid analysis of a hydrolyzed sample on a Beckman 6300 Amino Acid Analyzer with a System Gold enhancement. The amino acids used were judged pure by amino acid analysis and by elemental analysis.

EXAMPLE 1

Spectral Changes of D-Amino Acid
Transaminase
Induced by L-Amino Acids

In Fig. 1 the molar extinction coefficient of the enzyme is given as the dimer. The enzyme (0.25 mg/ml) was in 0.1 M Tris(hydroxymethyl) amino methane, (Tris) HCl, pH 7.5, containing 2 mM Ethylenediaminetetracetic acid (EDTA) and had been previously dialyzed against the same buffer to remove the excess of PLP. For the D-amino acid transaminase from the thermophilic Bacillus, the absorption spectrum displayed two bands in the wavelength region corresponding to the absorbance of the cofactor - at 420 and 338 nm. The absorption band at 420 nm corresponds to the pyridoxal 5'-phosphate (PLP) form of the enzyme. Addition of small amounts of D-alanine causes a shift in the spectrum from 420 nm with a corresponding increase in the 338 nm band, which has been assigned to the PMP form of the cofactor (Fig. 1). This effect is maximal at a concentration of about 0.5 mM D-alanine. A similar but less dramatic shift was also observed when the L-alanine was added to the enzyme (Fig. 1). Different batches of L-alanine from two different sources gave the same result.

TABLE I

EFFECT OF D- AND L-AMINO ACIDS ON THE SPECTRAL PROPERTIES OF D-AMINO ACID TRANSAMINASE

Amino Acid[a]	Relative Spectral Shift[b] from 420nm to 338 nm (%)
D-Alanine	100
L-Alanine	47
D-Glutamate	100
L-Glutamate	17
D-alpha-Aminobutyrate	100
L-alpha-aminobutyrate	0

[a] The degree of change was noted with 12.4 mM of each amino acid tested.

[b] The maximum decrease in the absorption at 420 nm attained by saturating concentrations of D-alanine was taken as 100% spectral shift as described in (Martinez del Pozo, A., et al (1989) Biochemistry 28:510-516, incorporated herein by reference). This absorbance band and the one at 338 nm due to the PMP form of the enzyme were the only spectral changes from 300-550 nm.

EXAMPLE 2

Detection of Formation of Pyruvate from L-Alanine

In Fig. 2 the reaction was carried out at 37°C in 0.35 M potassium phosphate, pH 8.5. Initially, the reaction mixture (1.45 ml) was made up of 35 mM L-alanine, 50 uM Nicotinamide adenine dinucleotide, reduced from (NADH), and 11 uM D-amino acid transaminase (DAT). AT the times indicated, the following reactants and enzymes were added sequentially, 50 ul of 94 µg/ml lactate dehydrogenase (LDG), 25 µl of 1 M L-alanine, 50 ul of 1 M D-alanine, and 1 µl of 0.1 M alpha-ketoglutarate. In the coupled enzyme assay depicted in Fig. 2, at the initial time the reactants were L-alanine, D-amino acid transaminase and NADH. Upon addition of lactate dehydrogenase, there was an immediate decrease in NADH. Upon addition of lactate dehydrogenase, there as an immediate decrease in NADH concentration corresponding to the formation of 13.7 µM pyruvate. The ratio of pyruvate formed to the amount of PLP (per subunit) is 1.25 ± 0.09 (average of three experiments). These results show that each of the subunits of the enzyme is functional and independently active and that L-alanine undergoes one turnover per subunit upon binding to D-amino acid transaminase. Subsequent addition of either L-alanine or D-alanine did not result in any further significant decrease in NADH (Fig. 2). This results confirms that the spectral shift was indeed indicative that the enzyme had been converted to the PMP form and that an alpha-keto acid was formed. The enzyme

remains fully active since addition of alpha-ketoglutarate to the cuvette (already containing D-alanine) causes an immediate and complete depletion of NADH in the coupled enzyme assay (Fig. 2).

EXAMPLE 3

Detection of Formation of D-Alanine from L-Alanine

In Fig. 3 the initial reactant concentrations were 17.2 mM L-alanine, 100 µM NADH, and 5,5 µM D-amino acid transaminase (DAT). After addition of the lactate dehydrogenase (LDH) (50 µl, see description of Fig. 2 in Example 2) pyruvate (10 µl of 0.1 M solution) was added after 3 min. Additional NADH (175 µl of 5 mM) was added to reduce the excess pyruvate. As soon as there was no further change in the absorbance at 340 nm, 10 µl of D-amino acid oxidase (DOX) (4.2 mg/ml), as well as the adequate amounts of Flavin adenine dinucleotide) (FAD) and catalase, were added to determine whether D-alanine has been formed.

After formation of 1 equivalent of pyruvate per enzyme subunit from L-alanine by a protocol similar to that described in Example 2, pyruvate was then added in excess to the PMP form of the enzyme in order to form alanine. After reduction of the excess pyruvate to lactate by lactate dehydrogenase and NADH, addition of D-amino acid oxidase brought about a decrease in the absorbance at 340 nm (Fig. 3). This result indicates that D-alanine had been formed. There is continued formation of D-alanine from L-alanine in the presence of excess L-alanine and excess pyruvate since the equilibrium is driven by the presence of lactate dehydrogenase and NADH.

The relative efficiency of D- and L-alanine as substrates for D-amino acid transaminase was assayed employing the lactic dehydrogenase-coupled assay. For L-alanine, concentrations of transaminase in the range of 0.1-0.5 mg/ml were used. The concentration dependence of the specific activity was also studied using different concentrations of enzyme and it was found to be linear. D-Amino acid transaminase catalyzes turnover of D-alanine at the rate of 1.22 x 104 units/μmol of enzyme. The corresponding rate with L-alanine is 4.2 units/μmol of enzyme

EXAMPLES 4-6

Determination of D-Alanine and D-Glutamate from L-Alanine and Alpha-Ketoglutarate

In Fig. 4 the elution profile was obtained by the injection of 170 nmol of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA)- derivatized amino acids after deproteinizing the reaction mixture as described below. The reaction mixture contained 10 umol of L-alanine, 10 μmol of alpha-ketoglutarate and 5.4 nmol of enzyme subunit.

Determination of the amount of D-alanine was performed by a procedure employing the HPLC separation of the diastereoisomeric amino acid derivatives after reaction with Marfey's reagent. After incubation of D-amino acid transaminase with L-alanine and pyruvate for 1 hr at 37°C, the reaction mixture was deproteinized, derivatized and chromatographed as described above in Materials and Methods. When the mole ratio of L-alanine to pyruvate was 100:1, 0.98 μmol of D-alanine was found. Assuming linearity for the reaction over that period of time, this represents a specific activity of 3.0 units/μmol of enzyme

subunit, which is of the same order of magnitude as found with the coupled enzymatic assay described above. If this same reaction was carried out but with a low concentration of alpha-ketoglutarate instead of pyruvate, only D-alanine is detected at about the same rate, 3.2 units/μmol of enzyme (Table II). In comparison the specific activity of the enzyme with D-amino acids is 1.22×10^4 units/μmol of enzyme. When limiting amounts of acceptor alpha-ketoacid were used, no D-glutamate was found even though an amount of D-glutamate as low as 1% of the total L-alanine added could have been detected. However, when the concentrations of L-alanine and alpha-ketoglutarate were made equimolar (10 μmol of each), D-glutamate was readily detectable in addition to D-alanine (Fig. 4, Table II). In this case, the rate of formation of D-glutamate was 3.8 units/μmol of enzyme, whereas the second equilibrium (See equation 4, below) occurs at a rate of 2.2 units/μmol enzyme subunit. In Fig. 4 the large peak corresponds to excess L-alanine and the smaller peaks were assigned to D-glutamate and to D-alanine by the use of the appropriate derivatized standards. The latter peaks were not found when D-amino acid transaminase was omitted from the incubation. The nearly equivalent amounts of D-amino acids indicate that there is an heterologous "racemization" which is catalyzed by D-amino acid transaminase as summarized by the equations:

3) L-alanine + alpha-ketoglutarate $\longrightarrow$ D-glutamate + pyruvate

4) D-glutamate + pyruvate $\rightleftharpoons$ D-alanine + alpha-ketoglutarate

When the amounts of alpha-ketoglutarate are limiting with respect to L-alanine in equation 3), reaction 4) is forced to the right because alpha-ketoglutatrate is needed for reaction 3). In this way the D-glutamate formed is rapidly converted to alpha-

ketoglutarate and only D-alanine is observed. Therefore, the amount of D-alanine formed is greater than the initial amount of alpha-ketoglutarate because of this recycling of alpha-ketoglutarate. On the other hand, when the amounts of alpha-ketoglutarate and L-alanine are equivalent in equation 3), then conditions are more favorable for equilibrium of reaction 4) and both D-glutamate and D-alanine are detected. The amounts of D-amino acids formed are much lower than the L-alanine used apparently because of the slow rate of reaction of the enzyme with L-amino acids. No production of L-glutamate was detected when alpha-ketoglutarate was the acceptor. If it were present, an amount of L-glutamate as low as 0.1% of the L-alanine could have been detected as determined with the appropriate standards.

TABLE II

Example	Substrates			Incubation Time	Products	
	L-Alanine	Pyruvate	Alpha-Ketoglutarate	D-Alanine	D-Glutamate	L-Glutamate
		µmoles		hrs	µmoles	
4	10.0	0.10	0.00	1	0.98	n.d.	n.d.
5	10.0	0.00	0.10	1	1.03	n.d.	n.d.
6	10.0	0.00	10.00	1	0.51	0.70	n.d.

[a] Experimental conditions: as described above with 5.4 nmole of monomeric enzyme subunit used for each Example; amounts of D-alanine and D-glutamate were determined with Marfey's reagent, as described above and illustrated in Fig. 4. n.d. = not detectable.

EXAMPLES 7 - 9

Conversion of Racemic DL-Alanine In The Presence of Alpha Ketoisovalerate

The conversion of racemic DL-alanine to D-valine in the presence of alpha-ketoisovalerate: The reaction mixture (2 ml) contained 0.1 M potassium phosphate pH 8.5, 5mM DL-alanine (10 µmoles), 50 mM (100 µmoles) of alpha-ketoisovalerate, 15mM NADH, 55 µg/ml of lactate dehydrogenase (LDH) and 2.1 µM of D-amino

acid transaminase. The reaction mixture was incubated for 16 and 62 hours at room temperature. The samples were then deproteinized and derivatized with Marfey's reagent to determine which isomer of valine was formed. The HPLC resolution was performed with the same column used for separation of the glutamate isomers described above and equilibrated in the Buffer A. The FDAA-amino acid derivatives were eluted with a three-step linear gradient of buffer B (50% v/v n-propanol in 0.1% v/v trifluoroacetic acid). The gradient was from 0-12% buffer B for 10 min, then from 12-15% for 60 min and finally from 15-50% of B for 10 min. The results of these Examples are shown in Table III. There was a 90% conversion to D-valine. No detectable L-valine was formed.

EXAMPLES 10-12

Conversion of Racemic DL-Alanine In the Presence of Alpha Ketoisocaproate

The conversion of racemic DL-alanine to D-leucine in the presence of alpha-ketoisocaproate: The reaction mixture (2 ml) contained 0.1 M potassium phosphate pH 8.5, 5mM DL-alanine (10 μmoles), 50 mM 100(μmoles) of alpha-ketoisocaproate, 15mM NADH, 55 μg/ml of LDH and 2.1 μM of D-amino acid transaminase. The reaction mixture was incubated for 16 and 62 hours at room temperature. The samples were then deproteinized and derivatized with Marfey's reagent to determine which isomer of leucine was formed. HPLC resolution was performed with the same column used for separation of glutamate isomers described above and equilibrated in the buffer A. The FDAA-amino acid derivatives were eluted with a three-step linear gradient of buffer B. The

gradient was from 0-12% buffer B for 10 min., then from 12-15% for 60 min. and finally from 15-50% of B for 10 min. The results of these Examples are provided in Table III.

TABLE III

Example	Substrates		Incubation Time	Products			
	DL-Alanine[b]	Alpha-Ketoisovalerate		D-Alanine	L-Alanine	D-Valine	L-Valine
	µmoles		hrs	µmoles			
7	10.0	100.0	0	5.00	5.00	n.d.	n.d.
8	10.0	100.0	16	n.d.	3.79	6.90	n.d.
9	10.0	100.0	62	n.d.	0.95	8.61	n.d.

	DL-Alanine[b]	Alpha-Ketoisocaproate		D-Alanine	L-Alanine	D-Leucine	L-Leucine
	µmoles		hrs	µmoles			
10	10.0	100.0	0	5.00	5.00	n.d.	n.d.
11	10.0	100.0	16	n.d.	3.46	5.93	n.d.
12	10.0	100.0	62	n.d.	1.26	8.35	n.d.

[b] These reactions were carried out at 25°C as described above with NADH and lactate dehydrogenase added to maximize the conversion to product. After the extended incubations the samples were deproteinized and an aliquot was derivatized with Marfey's reagent as described above. n.d. = not detectable.

WHAT IS CLAIMED IS:

1. A method for synthesizing a D-alpha-amino acid which comprises contacting under reaction conditions an alpha-keto acid corresponding to the D-alpha-amino acid to be synthesized, and a source of an amino group comprising an L-alpha-amino acid or a DL-alpha-amino acid mixture, in the presence as catalyst of D-amino acid transaminase in pyridoxal 5′ phosphate form.

2. The method according to claim 1 wherein the D-alpha-amino acid synthesized is D-alanine and the alpha-keto acid is pyruvate acid.

3. The method according to claim 1 wherein the D-alpha-amino acid synthesized is D-glutamic acid and the alpha-keto acid is alpha-ketoglutarate.

4. The method according to claim 1 wherein the D-alpha-amino acid synthesized is D-valine and the alpha-keto acid is alpha-ketoisovalerate.

5. The method according to claim 1 wherein the D-alpha-amino acid synthesized is D-leucine and the alpha-keto acid is alpha-ketoisocaproate.

6. The method according to any one of claims 1, 2, 3, 4 or 5 wherein the source of amino group comprises L-alanine or DL-alanine.

7. A method for synthesizing a D-alpha-amino acid which comprises contacting under continuous or batch reaction conditions, an alpha-keto acid corresponding to the D-alpha-amino acid to be synthesized, and a source of an amino group comprising an L-alpha-amino or a DL-alpha-amino acid mixture, in the presence as catalyst of D-amino acid transaminase in pyridoxal 5' phosphate form; said continuous or batch reaction conditions including means for driving equilibria towards production of the D-alpha-amino acid.

8. The method according to claim 7 wherein the D-alpha-amino acid synthesized is D-leucine and the alpha-keto acid is alpha-ketoisocaproate.

9. The method according to claim 7 wherein the D-alpha amino acid synthesized is D-valine and the alpha-keto acid is alpha-ketoisovalerate.

10. The method according to any one of claims 7, 8 or 9 wherein the source of amino group comprises L-alanine or DL-alanine.

11. The method according to anyone of claims 7, 8 or 9 wherein the means for driving equilibria towards production of the D-alpha-amino acid comprises means for reducing the concentration of alpha-keto acid corresponding to the source of amino group.

12. The method according to claim 11 wherein the means for reducing the concentration of alpha-keto acid corresponding to the source of amino group comprises adding nicotinamide adenine dinucleotide, reduced form and lactate dehydrogenase.

13. The method according to claim 11 wherein:

the contacting is under continuous conditions comprising:

(A) linking the catalyst to a resin column to form a catalyst-resin column;

(B) subjecting the catalyst-resin column to the source of an amino group to form a pyridoxamine 5′ phosphate catalyst-resin column; and

(C) passing the alpha-keto acid corresponding to the D-alpha-amino acid to be produced over the pyridoxamine 5′ phosphate-catalyst resin column; and,

the means for reducing a concentration of alpha-keto acid corresponding to the source of amino group comprises a physical separation wherein the alpha-keto acid corresponding to the source of amino group is eluted from the catalyst-resin column in step (B).

14. The method according to claim 11 wherein:

the contacting is under batch conditions comprising:

(A) linking the catalyst to a resin column to form a catalyst-resin column;

(B) subjecting the catalyst-resin column to the source of an amino group to form a pyridoxamine 5′ phosphate-catalyst-resin column; and

(C) soaking the pyridoxamine 5′ phosphate-catalyst resin column in the alpha-keto acid corresponding to the D-alpha-amino acid to be produced; and,

the means for reducing a concentration of alpha-keto acid corresponding to the source of amino group comprises a physical separation wherein the alpha-keto acid corresponding to the source of amino group is eluted from the catalyst-resin column in Step (B).

15. The method according to claim 12 wherein the source of amino group comprises L-alanine or DL-alanine.

16. The method according to claim 13 wherein the source of amino group comprises L-alanine or DL-alanine.

17. The method according to claim 14 wherein the source of amino group comprises L-alanine or DL-alanine.

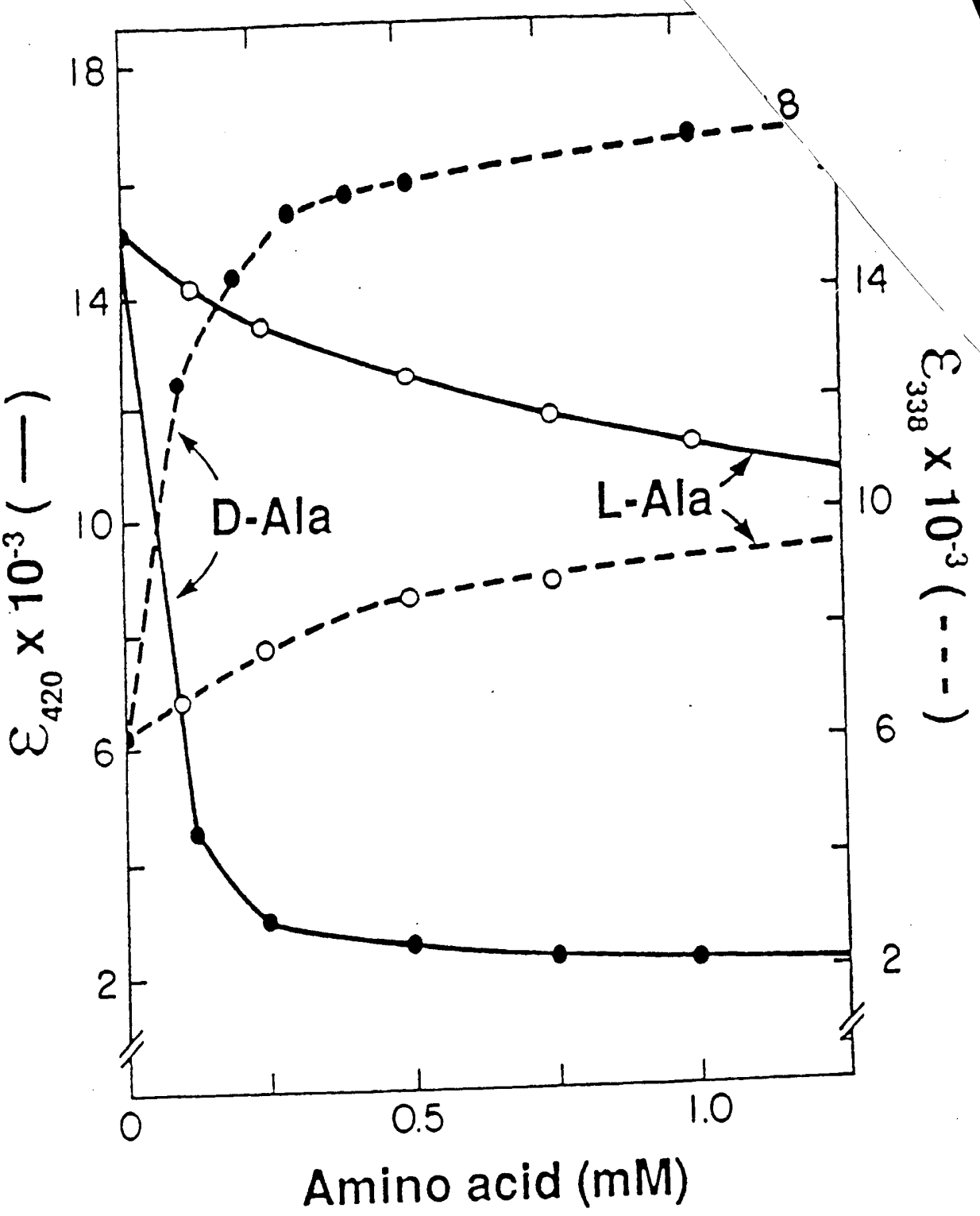
18
14
10
6
2
8
14
10
6
2
0
0.5
1.0
D-Ala
L-Ala
ε_{420} x 10^{-3} (—)
ε_{338} x 10^{-3} (- - -)
Amino acid (mM)

FIG. 1

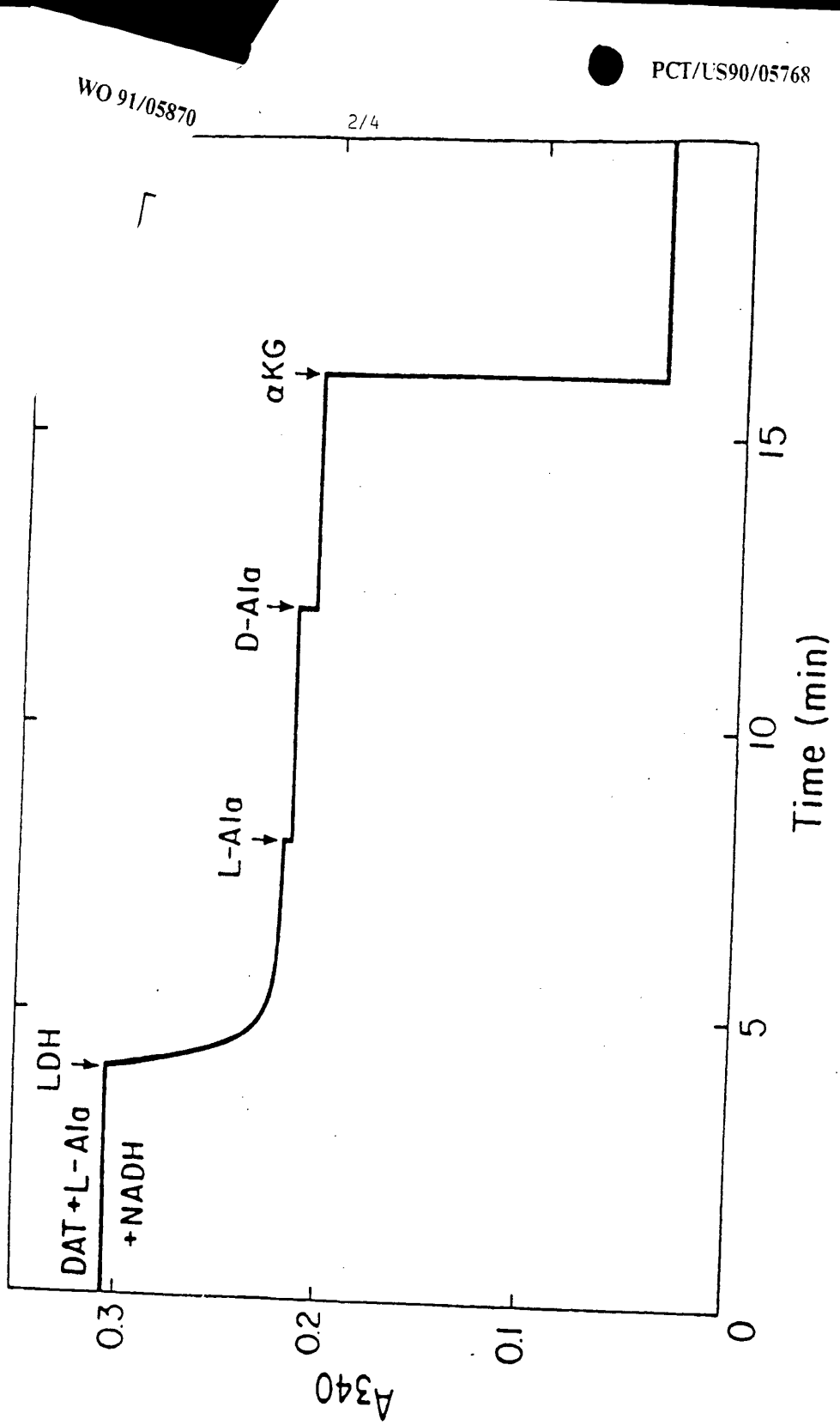

FIG. 2

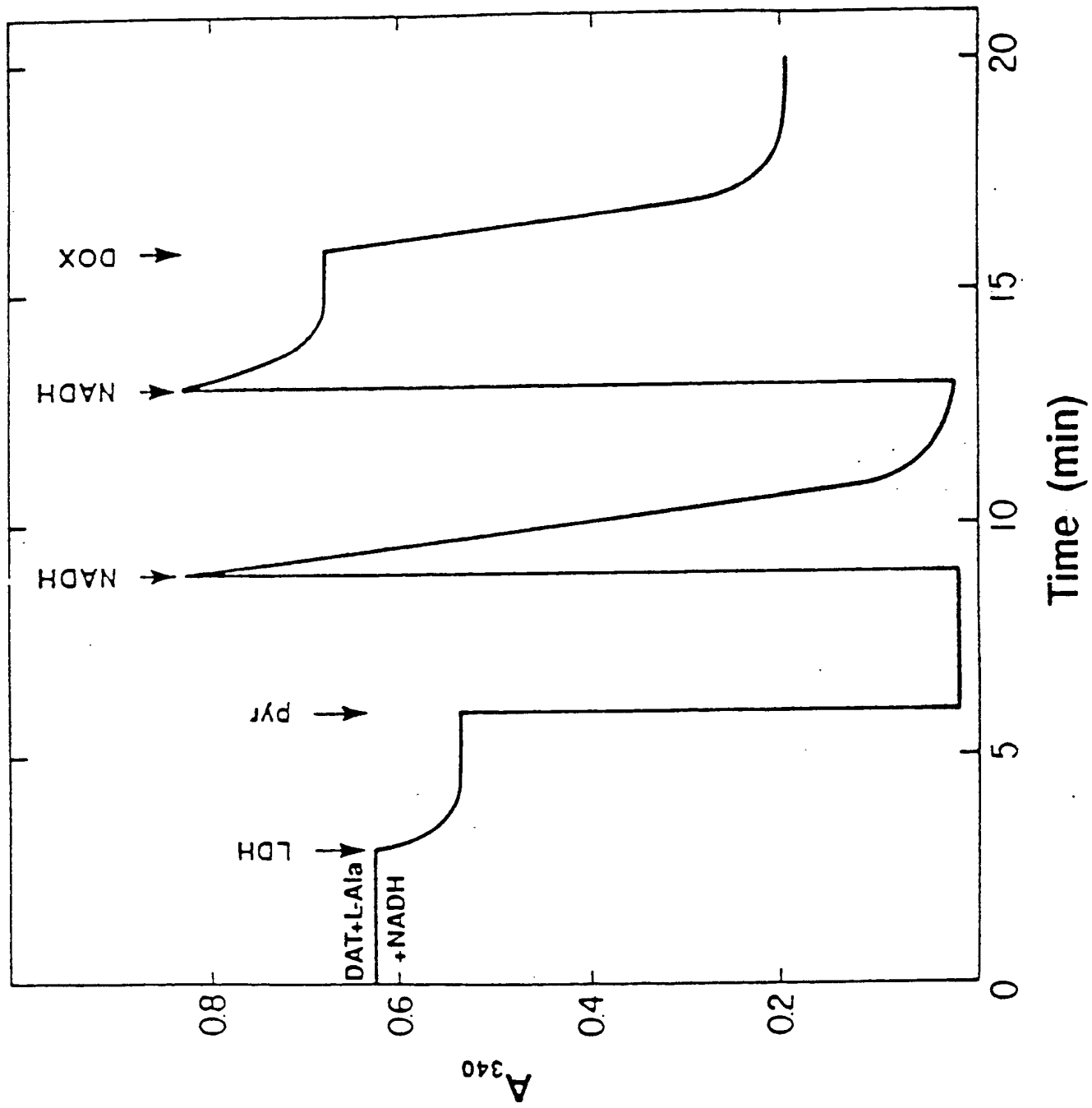
DOX
NADH
NADH
pyr
LDH
DAT+L-Ala
+NADH
0.8
0.6
0.4
0.2
A340
0
5
10
15
20
Time (min)

FIG. 3

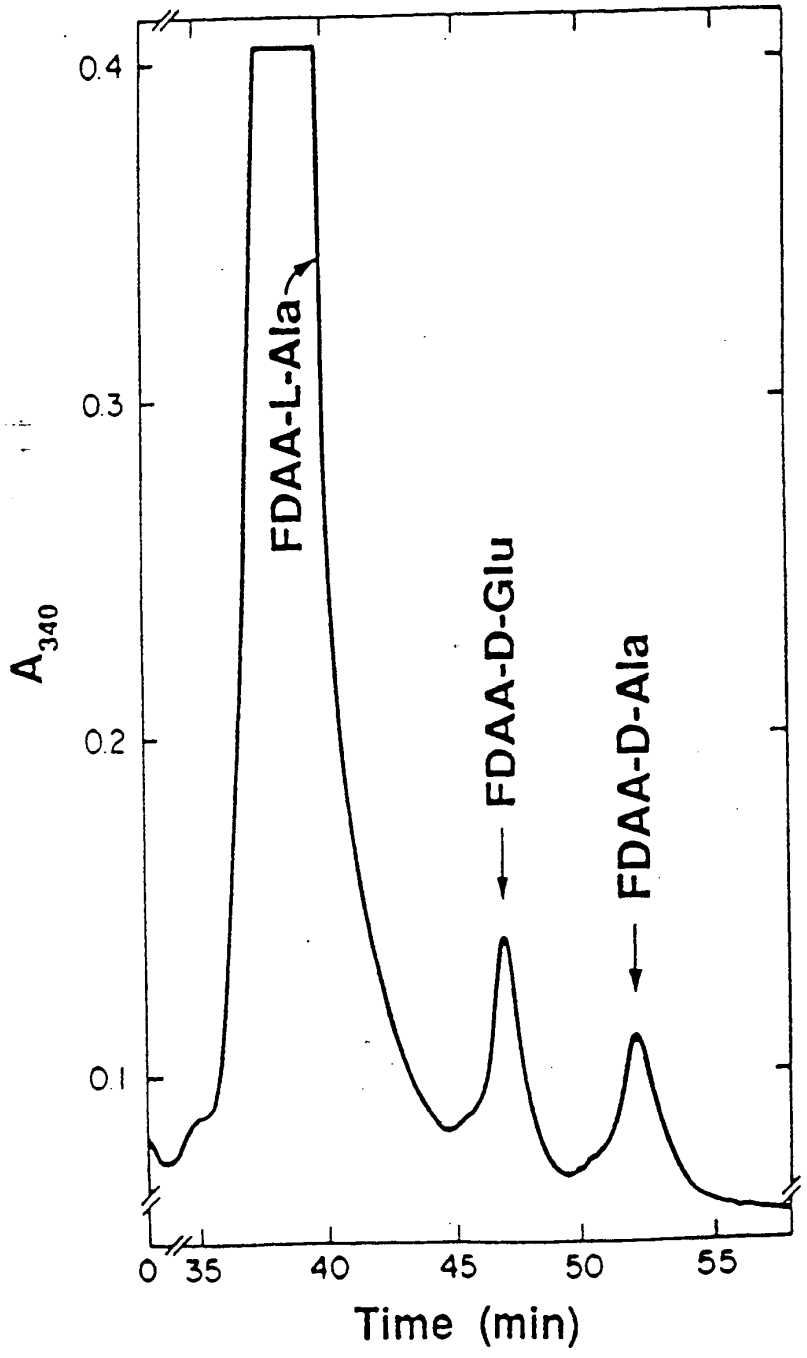
0.4
0.3
0.2
0.1
A_{340}
FDAA-L-Ala
FDAA-D-Glu
FDAA-D-Ala
0
35
40
45
50
55
Time (min)

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/05768

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 13/04,13/14,13/06,13/08; C12N 9/10
U.S.: 435/106,110,115,116,193,280

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/106,110,115,116,193,280

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

CAS ONLINE, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,X	The Journal of Biological Chemistry, Volume 264, No. 30, issued 25 October 1989, A. Martinez del Pozo, et al., "Stereospecificity of Reactions Catalyzed by Bacterial D-Amino Acid Transaminase", pages 17784-17789.	1-17
P,Y		1-17
X	Chemical Abstracts, Volume 108, No. 13, issued 28 March 1988, Soda et al., "Manufacture of D-amino acids from ketocarboxylic acids by enzymes" see page 492, column 2, abstract no. 110845K, Japan Kokai Tokkyo Koho JP62,205,790.	1-17
Y		1-17
Y	US, A, 4,826,766 (Rozzell) 02 May 1989, see entire document, especially columns 5 and 6.	7-12 and 15

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
04 January 1991	06 FEB 1991
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	C. M. GECKLE

Form PCT/ISA/210 (second sheet) (May 1986)